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Detection of signal recognition particle (SRP) RNAs in the nuclear ribosomal internal transcribed spacer 1 (ITS1) of three lineages of ectomycorrhizal fungi (Agaricomycetes, Basidiomycota)

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Abstract

During a routine scan for Signal Recognition Particle (SRP) RNAs in eukaryotic sequences, we surprisingly found in silico evidence in GenBank for a 265-base long SRP RNA sequence in the ITS1 region of a total of 11 fully identified species in three ectomycorrhizal genera of the Basidiomycota (Fungi): *Astraeus*, *Russula*, and *Lactarius*. To rule out sequence artifacts, one specimen from a species indicated to have the SRP RNA-containing ITS region in each of these genera was ordered and re-sequenced. Sequences identical to the corresponding GenBank entries were recovered, or in the case of a non-original but conspecific specimen differed by three bases, showing that these species indeed have an SRP RNA sequence incorporated into their ITS1 region. Other than the ribosomal genes, this is the first known case of non-coding RNAs in the eukaryotic ITS region, and it may assist in the examination of other types of insertions in fungal genomes.

Key words

SRP RNA, non-coding RNA, ribosomal RNA, internal transcribed spacer 1 (ITS1), ectomycorrhizal fungi

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Introduction

The nuclear ribosomal internal transcribed spacer (ITS) region is part of the ribosomal DNA cistron. The ITS region is transcribed together with the 18S, 5.8S, and 28S genes but removed in the post-transcriptional processing of the rRNA. The ITS region has three separate subregions: the ITS1, the 5.8S gene, and the ITS2. The ITS1 is situated between the 18S and the 5.8S genes, and the ITS2 is situated between the 5.8S and the 28S genes. The ITS region varies significantly in length among fungal species (Taylor and McCormick 2008, Tedersoo et al. 2015), and both of ITS1 and ITS2 form secondary structures with stems, bulges, and loops (Freire et al. 2012, Rampersad 2014). The secondary structure is important for correct processing of the rRNA; although the ITS1 and ITS2 regions are not expressed in the ribosome, there are constraints on the evolution of the ITS region, and it has both fast evolving and more conserved regions (Nazar 2004, Mullineaux and Hausner 2009). However, there is still much to learn about the function of the ITS region, especially for ITS1 (Rampersad 2014, Coleman 2015). The fast evolving regions of ITS has made it a cornerstone in species/genus-level phylogenetic inference in fungi and other organisms for more than 20 years, and it is the formal fungal barcode used for molecular species identification (Álvarez and Wendel 2003, Schoch et al. 2012).

One element that has never been implicated in the context of the eukaryotic rDNA cluster and ITS evolution is the existence of non-coding RNAs (ncRNA) other than the 18S, 5.8S, and 28S rRNAs. Based on the recent identification of a ubiquitous eukaryotic ncRNA in the fungal phylum Basidiomycota, viz. the Signal Recognition Particle RNA (SRP RNA; Dumesic et al. 2015), we discovered a ~265 bases long homologue of this gene in a set of fungal ITS1 sequences (Fig. 1). The SRP RNA is an essential component of the SRP, a ribonucleoprotein particle that co-translationally directs proteins to the endoplasmic reticulum (ER) membrane. The SRP RNA acts both as a scaffold for the SRP proteins and as a regulator of the SRP by mediating a global reorganization of the SRP in response to cargo binding (Rosenblad et al. 2009, Akopian et al. 2013).

A more thorough in silico analysis verified the presence of SRP RNAs in the ITS1 region of a total of 11 fully identified fungal species (separate Latin binomials) distributed over three lineages of ectomycorrhizal basidiomycetes (Boletales: *Astraeus* (1 species: *A. sirindhorniae*), Russulales: *Russula* (1 species: *R. olivacea*), and Russulales: *Lactarius* (9 species: *L. argillaceifolius*, *L. aspideus*, *L. brunneoviolaceus*, *L. luridus*, *L. nanus*, *L. pallescens*, *L. pseudouvidus*, *L. uvidus*, and *L. violascens*). The notion of an additional ncRNA element in the ITS1 region is novel and would seem – at least at a first glance – to compromise the function of the ITS1. Hypothetically, any of contamination, chimeric unions, or other laboratory or data analysis artifacts could explain this finding. In this study we apply DNA sequencing and bioinformatics to verify the presence of SRP RNA sequences in the ITS1 region of representatives of these fungi.

Materials and methods

SRP RNA bioinformatics

The bioinformatic analysis of non-coding RNAs such as the SRP RNA is not trivial, as the primary sequence may vary substantially as long as the secondary structure is preserved. To enable searches for SRP RNAs without requiring exact sequence matches across the full length of the SRP RNA, we used a secondary structure covariance model constructed from the full set of available ascomycete SRP RNAs with the basidiomycete SRP RNAs from Dumesic et al. (2015) added, as well as a second dataset containing all covariance models from Rfam (Nawrocki et al. 2014). These models were used in an INFERNAL v1.1 cmsearch (Nawrocki and Eddy 2013) run against flatfiles of the International Nucleotide Sequence Database Collaboration (INSDC; Nakamura et al. 2013; February 2015 release). After observing several highly significant matches to what seemed to be the fungal ITS1 region, we re-ran the search on the flatfiles from the manually curated fungal ITS database UNITE (Abarenkov et al. 2010; release 2015-08-01) using ITSx 1.0.11 (Bengtsson-Palme et al. 2013) to identify the ITS1 region. A total of 63 matches and 11 fully identified species from three ectomycorrhizal basidiomycete lineages were recovered (Suppl. material 1). All matches were examined manually to verify that they displayed all the universally conserved motifs and nucleotides. The sequences were found to stem from more than 20 different published and unpublished studies. The fact that these sequences had been found multiple times independently is highly suggestive of technically sound, authentic sequence data (Nilsson et al. 2012), but to further confirm the authenticity of the sequences we re-sequenced one herbarium specimen from each of these lineages, either from the original material or from other conspecific specimens.

PCR and sequencing

To rule out systematic PCR artifacts as sources of false positives in the bioinformatics analyses, we retrieved the original, or conspecific, specimens underlying one representative from each of the genera (Table 1): the conspecific collection *Astraeus sirindhorniae* MA-Fungi 47735 (collected in the Philippines; herbarium MA), the authentic collection *Lactarius luridus* TU118993 (collected in Estonia; herbarium TU), and the authentic collection *Russula olivacea* TU101845 (collected in Estonia; herbarium TU). We specifically sought to use a different primer pair combination and PCR conditions than did the original sequence authors in a further attempt at generalizing our findings. For *A. sirindhorniae*, DNA extractions, PCR reactions, and sequencing were performed as described in Martín and Winka (2000), however using DNeasy Plant Mini Kit (Qiagen) with overnight incubation for DNA extraction. Primers used for amplification were ITS5/ITS4 (White et al. 1990; Suppl. material 1). For the specimens of

Lactarius and *Russula*, the DNA was extracted and amplified using the primers ITS1f and ITS4b following Anslan and Tedersoo (2015). Sequences were edited and assembled using Sequencher 4.2 (Gene Codes, Ann Arbor). All sequences were examined for sequence quality following Nilsson et al. (2012). Chimera detection was undertaken using UCHIME (Edgar et al. 2011) and the UNITE chimera reference dataset (Nilsson et al. 2015; release 2015-03-11).

Results

The ITS sequences recovered from the sequencing round passed all quality control measures we exercised. In addition, no sequence was found to have the multiple DNA ambiguity symbols suggestive of the presence of several information-wise distinct ITS copies in the individuals at hand (Hyde et al. 2013). The resulting sequences manifested the SRP RNA sequence in the ITS1 region of all three re-sequenced lineages. The two authentic specimens of *Russula* and *Lactarius* produced identical ITS sequences to those already extant. The sequence from the conspecific *Astraeus sirindhorniae* specimen differed by three bases from the extant sequence, which is well within the expected intraspecific variation when conspecific isolates are compared across geographical distances (Thailand and the Philippines in this case). The sequences were deposited in the INSDC as accessions KU356730-KU356732.

SRP RNA-containing sequences of *Russula* and *Lactarius* were found to have an average length of some 890 bases; the corresponding average length for the SRP RNA-containing *Astraeus* sequences was 840 bases. When using BLAST to find the most similar sequences of *Russula*, *Lactarius*, and *Astraeus* that did not contain the SRP RNA, we found that their ITS region was on average 616 bases (*Russula*), 644 bases (*Lactarius*), and 620 bases (*Astraeus*). This corresponds well to the length of the SRP RNA (~265 bases) for all of *Astraeus*, *Russula,* and *Lactarius*, allowing for some few bases of divergence considering the cross-species comparison. The distances between the SRP RNA and the surrounding genes 18S and 5.8S were almost the same within each lineage, but differed somewhat among the three lineages: 80 and 174 bases (*Lactarius*), 150 and 80 bases (*Russula*), and 55 and 132 bases (*Astraeus*).

Discussion

The finding that ncRNAs are located in tandem is not novel. Apart from the highly conserved nuclear rDNA cluster, some ncRNAs have been found to cluster in several protist species, e.g., SRP RNA together with U6 snRNA, 5S rRNA, SL RNA, and tRNAs in dinoflagellates (Zhang et al. 2013). Regarding the transcription of the SRP RNA in fungi, the transcriptional promoters of the SRP RNA in *Saccharomyces cerevisiae* (the TFIIIC-binding A- and B-box) are internal, and the SRP RNAs have a polypyrimidine termination sequence similar to other RNA polymerase III genes. Although

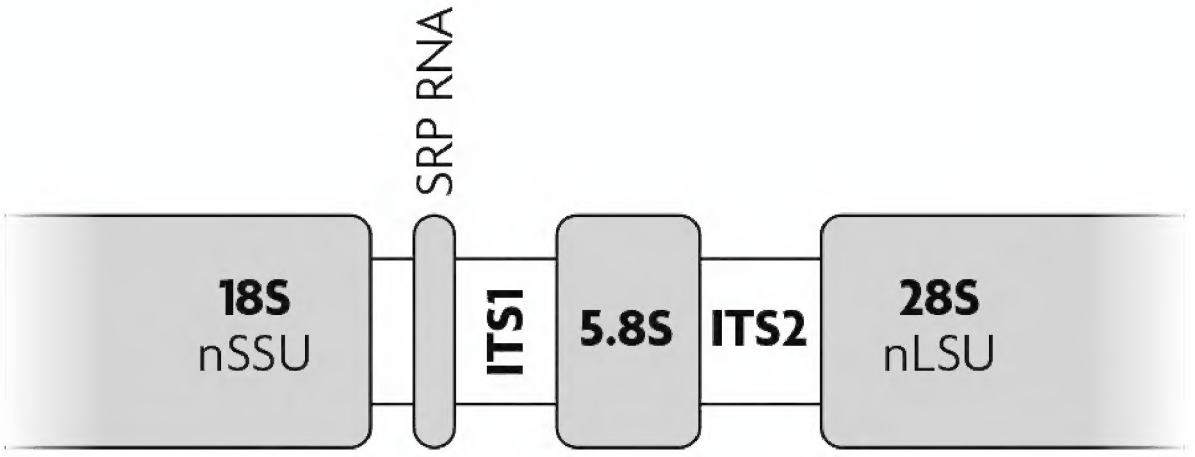

Figure 1. Schematic illustration of the fungal ITS region and neighboring rDNA genes. The subregions ITS1, 5.8S, and ITS2 of the ITS region are indicated along with the SRP RNA in the first part of the ITS1. The absolute positions of the subregions and the SRP RNA are provided in Suppl. material 2.

yeast SRP RNA also has an upstream TATA box – a motif we could not clearly identify in our alignments – inactivation of this region does not result in a significant effect on transcription (Dieci et al. 2002). Therefore it is possible that our identified SRP RNAs could be transcribed independently of the transcription of the rDNA cluster. If so, the SRP RNAs found in the ITS1 region most probably are fully functional, but there may be another copy in the genome that constitutes the major transcript. Whether the SRP RNA could be a product of the rDNA processing seems less likely. Although the A_3 cleavage site that is the closest to the 5.8S should be downstream of the SRP RNA 3' end since the distance between the SRP RNA and the 5.8S is at least 80 nucleotides in the 63 sequences, the SRP RNA needs correct 5' and 3' ends to fold into the proper secondary structure. Therefore the processing of the tricistronic ribosomal transcript most probably leads to non-functional SRP RNA. The surprising lack of mutations, as compared to other identified basidiomycete SRP RNAs (Suppl. material 4), could be explained not only by the insertion being a recent event, but also by the need to preserve the secondary structure of the SRP RNA region and thus the remaining parts of ITS1.

The three species from which the SRP RNA was recovered are all ectomycorrhizal basidiomycetes and come from two different orders and two different families. Two of these lineages are closely related (*Russula* and *Lactarius*, both in Russulaceae (Russulales)); the third one – *Astraeus sirindhorniae* (Boletales) – comes from the same subphylum (Agaricomycotina) as the former two. Even so, the Russulales and the Boletales are separate orders, such that the presence of SRP RNAs in these fungi must be considered independent gains. In the case of *Russula* and *Lactarius* – two very speciose genera – the vast majority of the known species do not have the SRP RNAs in their ITS1. Similarly, none of the other species in *Astraeus* treated by Phosri et al. (2014) were found to have the SRP RNA. It would appear far more realistic to view these SRP RNAs as independent insertion events than as a plesiomorphic ITS state where the ancestor contained the SRP RNA, but where all species except the few considered here lost it (Miller et al. 2006; Suppl. materials 1–4). Indeed, we found no evidence for SRP RNA in the ITS region of any other fungus.

Table 1. Data on the underlying specimens and PCR primers. The already sequenced specimens of *Russula* and *Lactarius* were re-sequenced with a different primer pair compared to the extant sequences. Our Philippines specimen of *Astraeus sirindhorniae* had never been sequenced before, but we used a different primer pair compared to the *Astraeus sirindhorniae* sequence generated by Phosri et al. (2014) from a Thailand collection. Primer sequences are available in Suppl. material 1B.

Species	Original entry	Resequenced entry	Specimen	Herbarium	Country	Original primers	Resequencing primers	ITS1	5.8S	ITS2
Astraeus sirindhorniae	(not sequenced before)	KU356730	MA-Fungi 47735	Madrid	Phillipines	(not sequenced before)	ITS5 / ITS4	1–442	443–444	-
Lactarius luridus	UDB023551 (UNITE)	KU356731	TU118993	Tartu	Estonia	ITS0F / LB-W	ITS1F / ITS4b	1–482	483–640	641–896
Russula olivacea	UDB016000 (UNITE)	KU356732	TU101845	Tartu	Estonia	ITS0Ft / LB-W	ITS1F / ITS4b	1–457	458–615	616–891

The three previously identified SRP RNAs in the Russulales are not located in or close to the rDNA cluster (Dumesic et al. 2015), and we argue that the SRP RNA must be considered as an independently inserted element in these ITS1 sequences. Although this does not cause any problems in terms of molecular identification of these species, it does present a potential difficulty to the uncritical use of ITS sequences in phylogenetic inference in these fungal lineages. Under the assumption that the SRP RNA is found in the exact same position in the ITS1 region among species, the sequences could still be aligned jointly as long as the SRP RNA part is kept as a separate element in the multiple sequence alignment. Any failure to realize that the SRP RNA should be treated as a separate element to be scored as gaps in species that do not have the SRP RNA is certain to give rise to very noisy multiple sequence alignments and skewed inferences of phylogeny. In other words, there is a risk that alignment tools will try to align other parts of the ITS1 region onto the SRP RNA part in large alignments, which would violate homology assumptions. We briefly examined whether several of the most commonly used alignment programs were able to recognize the unique nature of the SRP RNA and not try to stack other parts of the ITS1 onto the SRP RNA. The results were generally encouraging as long as the number of non-SRP RNA containing species was kept reasonably low, with only minor manual adjustments needed a posteriori. In the worse situation where the SRP RNA insertion is not found in the exact same position across species, it will not be possible to maintain position homology in the multiple sequence alignment. In that scenario, the sequences containing the SRP RNA must be excluded from the alignment process, or the SRP RNA element must be removed. Interestingly, Eberhardt (2002) reported an unexpected 250-base insertion in the ITS1 of *Russula olivacea* – one of the species examined in the present study – and chose to exclude it from her multiple sequence alignment due to alignment difficulties. In hindsight it seems probable that this 250-base region indeed represents the SRP RNA. As demonstrated by the Eberhardt (2002) example, there is widespread (although not necessarily universal) awareness of the importance of examining multiple sequence alignments manually before they are put to scientific use. However, the increasing use of fully automated solutions to data harvesting and phylogenetic inference may present a concern here (cf. Antonelli et al. 2014).

Our findings are not without potential shortcomings though. The number of ITS copies per fungal cell can approach 200 or more (Bellemain et al. 2010; Black et al. 2013). Whereas the process of concerted evolution is thought to homogenize the array of ITS copies (Álvarez and Wendel 2003), it is not uncommon to find evidence of two or more distinct ITS copies during sequencing work (Hyde et al. 2013). A recent pyrosequencing-based study found evidence for multiple, information-wise distinct ITS copies in 3–5% of the 99 examined species of Ascomycota and Basidiomycota (Lindner et al. 2013). The extent to which the three species examined here contain multiple distinct ITS copies is unknown but may well be low, given that we obtained single, clean PCR products and sequence chromatograms for all three species. Even so, it is conceivable that we – much like the original sequence authors – in fact amplified a rare and perhaps non-functional ITS copy. Although this would not disqualify our

finding of an SRP RNA in the fungal ITS region, it would raise questions regarding whether the inclusion of the SRP RNA ruined the function of this particular ITS copy, essentially rendering the corresponding rRNA non-functional. In either case, we view this multiple-copy scenario as unlikely given our consistent obtainment, and the more than 20 independent recoveries, of the SRP RNA-containing ITS1 sequences.

Unfortunately, none of the species of the present study have a complete genome published, so a detailed analysis of the SRP RNA in the context of the genomes of these and closely related fungi will have to wait. That said, the trend that published fungal genomes tend to come without the ribosomal operon for reasons of convenience is most unfortunate (Schoch et al. 2014). We join the barcoding community in extending a plea that whenever a genome is sequenced and assembled, the ribosomal operon should be assembled into the genome as a part of that process. If this undertaking proves to be too complex, then at least the full ribosomal operon should be bundled with the genome, even if its assembly into the genome cannot be accomplished.

Conclusions

We found evidence of Signal Recognition Particle (SRP) RNAs in the ITS1 region of a total of 11 fully identified species in three ectomycorrhizal genera: *Astraeus*, *Russula*, and *Lactarius*. Other than the ribosomal genes, this is the first known case of non-coding RNAs in the fungal ITS region. Our finding is small step towards explaining the many insertions found throughout fungal genomes, and it adds a new element to the field of fungal ITS evolution.

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Supplementary material 1

Output from cmsearch and primers used

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Data type: text/computer output

Explanation note: A) The output from cmsearch showing all 63 relevant matches to the three ectomycorrhizal lineages. B) Detail of the primers used to re-amplify the specimens.

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Supplementary material 2

ITS multiple sequence alignment

Authors: Magnus Alm Rosenblad, María P. Martín, Leho Tedersoo, Martin Ryberg, Ellen Larsson, Christian Wurzbacher, Kessy Abarenkov, R. Henrik Nilsson

Data type: text/DNA sequence data

Explanation note: A multiple sequence alignment in the NEXUS format (Maddison et al. 1997) comprising all 63 matching ITS sequences, plus the three newly generated ones (KU356730, KU356731, and KU356732). The alignment was produced in MAFFT without manual adjustment (Katoh and Standley 2013). The alignment is composed of partial nSSU (bases 1-34 in the alignment), the full ITS1 (bases 35-678), the full 5.8S (bases 679-838), the full ITS2 (bases 839-1395), and partial nLSU (bases 1396-end). The SRP RNA occupies position 203-474 in the alignment. The alignment is provided for overview purposes only; the two-order nature of the taxa (Boletales and Russulales) coupled with the high variability of the ITS region jointly mean that the alignment will not be suited for phylogenetic inference.

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Supplementary material 3

ITS/SRP RNA multiple sequence alignment

Authors: Magnus Alm Rosenblad, María P. Martín, Leho Tedersoo, Martin Ryberg, Ellen Larsson, Christian Wurzbacher, Kessy Abarenkov, R. Henrik Nilsson

Data type: text/DNA sequence data

Explanation note: Multiple sequence alignment comprising the 63 public ITS1 sequences with SRP RNA found in them, the three newly generated sequences, and the SRP RNA sequences from Dumesic et al. (2015) (*Stereum hirsutum*, *Heterobasidion irregulare*, and *Heterobasidion annosum*).

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Supplementary material 4

SRP RNA multiple sequence alignment

Authors: Magnus Alm Rosenblad, María P. Martín, Leho Tedersoo, Martin Ryberg, Ellen Larsson, Christian Wurzbacher, Kessy Abarenkov, R. Henrik Nilsson

Data type: text/DNA sequence data

Explanation note: Multiple sequence alignment with the SRP RNA sequences of Dumesic et al. (2015; *Stereum hirsutum*, *Heterobasidion irregulare*, and *Heterobasidion annosum*) aligned to our newly generated ITS sequences of *Russula* and *Lactarius*.

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